DEVELOPMENT OF A BATH CHALLENGE SYSTEM TO STUDY COMPONENT CAUSES, AND PREVENTATIVE TREATMENTS, OF EPIZOOTIC ULCERATIVE SYNDROME (EUS) IN SNAKEHEAD FISH (*CHANNA STRIATA*).

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Declaration

This is to certify that the work submitted was carried out by the candidate.

Candidate's Signature……………………………………..

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ABSTRACT

A bath challenge system was developed to enable further investigation of the causative factors of EUS. This looked at water quality parameters as contributory factors, examining the effects of (i) low alkalinity, low hardness water, (ii) acidified low alkalinity, low hardness water, and (iii) tapwater.

Fish were exposed to sporulating fungal wads of *Aphanomyces invadans*, after initial exposure to water treatments for 24 hours. Fish were examined over a 30 day period for signs of lesions. Once lesions were observed fish were sampled, and the area below the lesion was examined histologically for the presence of an intramuscular fungal invasion. The study of acidified and distilled water as causal factors in EUS revealed there was no difference between these treatment groups and tapwater. It was thought however that low alkalinity and hardness do play a part in increasing susceptibilty of snakehead (*Channa striata*) to EUS, as tapwater also had alkalinity and hardness values that were considered low. Behavioural observations were also highlighted as potential factors.

An *in vitro* method of screening potential fungicides revealed that from a number of compounds (D-limonene, E-Z-Mulse™, a D-limonene/ E-Z-Mulse™ emulsion, a commercial neem seed extract, a coarse neem seed extract, and a coarse neem leaf extract), only E-Z-Mulse™ and the commercial neem extract showed

any reasonable activity against *A. Invadans* mycelium, or the secondary zoospore stage.

The combination of the developed bath challenge and screened fungicides was used to study the efficacy of a number of potentially active fungicides. These compounds included calcium oxide (CaO), CIFAX (a claimed preventative and curative treatment of EUS), and the two highlighted compounds from *in vitro* screening, E-Z-Mulse™ and commercial neem extract. Abraded fish were exposed to sporulating wads of *A. invadans* and sampled after 5 days, with freshly abraded fish and mycelial wads added every 5 days, over a 15 days period. Water remained in the tanks for duration of the investigation to gauge efficacy of treatments. This exposure protocol failed to precipitate sufficient infection within controls to enable accurate examination of treatments.

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CHAPTER 1

1. Introduction

Epizootic Ulcerative Syndrome (EUS) is a seasonal epizootic disease affecting wild and farmed freshwater and brackishwater fish throughout Southeast Asia, Australia, Japan, and more recently, the Indian sub-continent and Pakistan. The disease is characterised by the presence of invasive *Aphanomyces invadans* infection and necrotising ulcerative lesions typically leading to a granulomatous response.

The occurrence of the disease in many species (Annex 1) has resulted in severe losses in established culture and capture fisheries in Asia and Australia for more than two decades, and has also limited the development of fisheries and aquaculture in parts of Asia.

EUS is thought to have first appeared in Thailand in 1981. Outbreaks appeared throughout the southern provinces, spreading to the central areas in 1982. It soon became the most significant epizootic in Thailand, greatly affecting snakehead culture, one of the most susceptible and economically important species (Annex 2), and also causing severe mortalities of wild and rice-field fish.

The first recognized account of an EUS-like condition occurred in Japan in 1971 (Egusa and Masuda, 1971). The disease was characterised by a

granulomatous response to invasive fungus, and was named mycotic granulomatosis (MG) (Miyazaki and Egusa, 1972). The following year, 1972, a disease outbreak in Queensland, Australia, affecting estuarine fish, particularly grey mullet *(Mugil cephalus)*, resulted in the spread of the disease (independantly named Red Spot Disease, RSD) to coastal tributaries and associated rivers in New South Wales (Callinan *et al.*, 1989), Northern Territories (Pearce, 1990), and Western Australia (Callinan, 1994). Since the first recorded outbreak of an EUS-like condition in Japan, other countries have reported similar dermal ulcer associated syndromes, resulting in large mortalities of fresh and brackishwater fish species. The disease has been seen to spread west across Asia, reaching the Indian sub-continent in 1988. Pakistan; 1996, has been the most recent country to be affected.

The spread of EUS has been so rapid, and the consequences so dramatic, that in recent meetings held by the European Union Scientific Sub-Committee on Animal Health, examination of the possible introduction of EUS to Europe has been addressed (Ahl, 1997), and also in the independent assessors report prepared for the Ministry of Agriculture Fisheries and Food (Slaski, 1999).

1.1 Aetiology

The occurrence of EUS- like ulcerative disease outbreaks in a range of habitats and species since 1971, has led to the isolation and identification of a number of associated pathogens. Some of these may indeed significantly contribute to pathogenesis in particular outbreaks, however only the presence of a single species of *Aphanomyces* "fungus", *Aphanomyces invadans*, has consistently been isolated from EUS-infected fish, and is regarded as the only definite cause of EUS identified and confirmed at present. A combination of causal factors have been suggested as constituting sufficient cause for the disease, not only the presence of *A. invadans* and a susceptible fish species. The other component causes suggested at present are (i) the exposure of the dermis by mechanical abrasion, (ii) the intramuscular injection of rhabdovirus T9412, and (iii) the sublethal exposure of fish to acid sulphate soil runoff water.

The causative *Aphanomyces* fungus has previously been named as *Aphanomyces piscicida* (Hatai, 1980), *Aphanomyces invaderis* (Willoughby *et al.*, 1995), and ERA (EUS-related *Aphanomyces* species, Lumanlan-Mayo *et al.*, 1997), isolated from Japan, Thailand, and Philippines, respectively. Recently, pathogenic isolates from these and other countries have been compared genetically (biochemical fingerprinting using pyrolysis mass spectra (PyMS) and molecular studies involving random amplification of polymorphic DNA), and shown to be very similar (Lilley *et al.*, 1997). Protein and carbohydrate electrophoresis banding patterns, growth characteristics, and

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chemical susceptibility, have all confirmed the isolates as being of the same species. As all isolates have been confirmed as conspecific, a single species name, *Aphanomyces invadans*, has been adopted.

Aphanomyces invadans is an aseptate fungi (Figure 1), producing two zoospore forms. Sporulating *A. invadans* cultures discharge primary zoospores from the mouth of terminal sporangia or from evacuation tubes. Within 12 hrs of sporangial development (at 22°C) the primary zoospores encyst to from clusters at the mouth of the sporangium which release the secondary form, subspherical, laterally biflagellate, free swimming infective secondary zoospores (Figure 2).
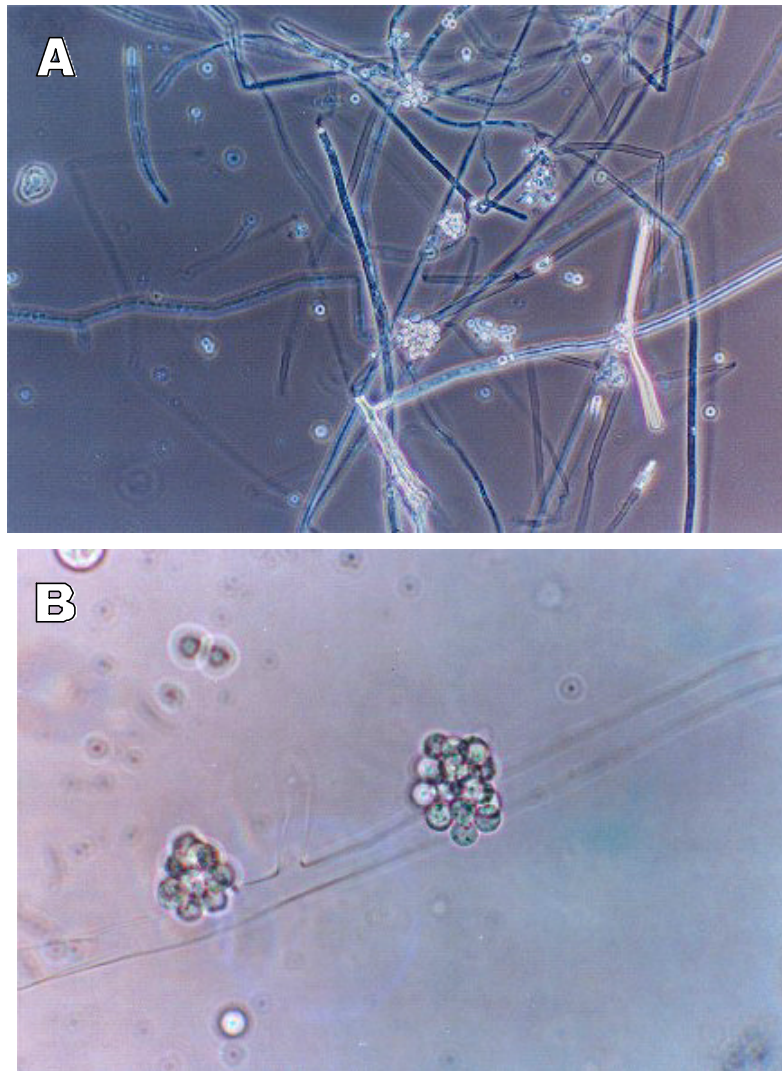
Figure 1 A, Fungal hyphae showing clusters of encysted primary zoospores of *Aphanomyces invadans* (40 X magnification). **B,** Achyloid clusters, hyphae and lateral evacuation tube of *Aphanomyces invadans*, (100 X magnification).
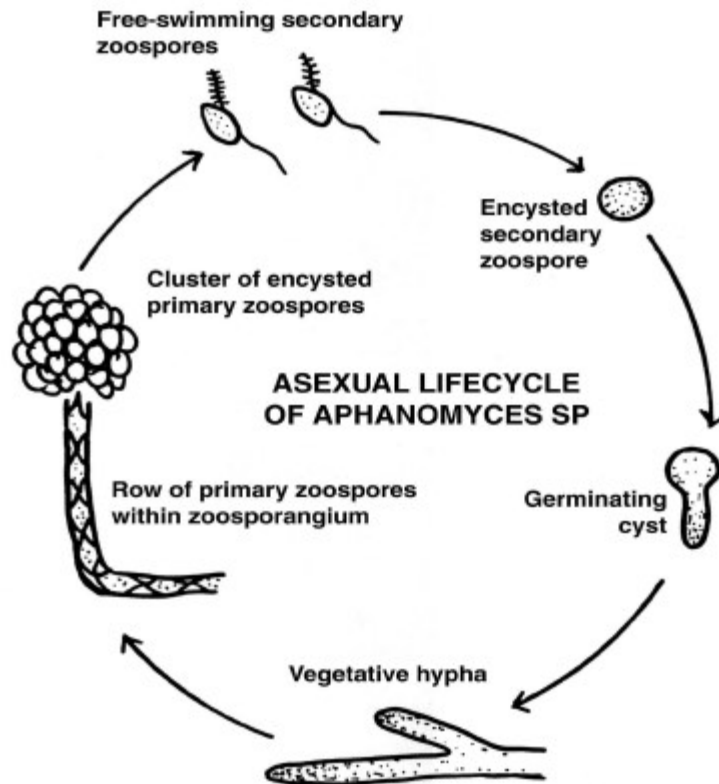
Fig 2 The asexual life cycle of *Aphanomyces invadans*.

(Adapted and reproduced by kind permission. Lilley, 1997)

1.2 Environmental factors

The possible role of environmental factors in outbreaks of EUS has been the subject of considerable discussion since the first outbreaks of EUS in Southeast Asia during the early 1980's. EUS outbreaks occur in a cyclical manner, and appear to have some correlation with diurnal temperature fluctuations, and periods of heavy rainfall. Water quality during outbreaks often shows decreased alkalinity, hardness and chloride concentrations (Phillips, 1994).

Outbreaks usually follow changes in water temperature, and periods of heavy rainfall. These two combined environmental factors can have markedly different effects on water quality and resulting physico-chemical properties depending on climate, geology, river systems and a host of other factors. No one environmental factor, or combination of factors can link outbreaks of EUS at all sites. For instance in Australia and the Philippines , EUS outbreaks have been associated with acidified runoff water from acid sulfate soil areas during periods of heavy rainfall (Sammut *et al.*, 1996) with highest prevalence recorded in tributaries draining acid sulphate areas. The interaction between rainfall, temperature, and acid-sulphate soils resulted in fish being sub-lethally exposed to acid water which caused dermal necrosis, and this is likely to have been sufficient to allow penetration by *A. invadans* and initiation of EUS. Outbreaks in Thailand, however, have not been associated with acidified runoff. Severity of outbreaks seems to be directly related to heavy rainfall, low

water temperature, and distinctly low alkalinity (3-20 mg/l $CaCO_3$)(Chinabut, 1994).

Temperature has long been correlated to incidence and severity of EUS outbreaks in all countries where the disease has been reported, and may in part explain the cyclical pattern of outbreaks. Decreased temperature impairs fish immune function, and consequently the ability to contain and inactivate the invasive fungus. Chinabut *et al.* (1995) demonstrated that snakehead (*Channa striata*) inoculated with *Aphanomyces invadans* succumbed to the disease when held at 19 ^{o}C, whereas those kept at 26 and 31 ^{o}C showed a more rapid cellular immune response and much lower mortality.

At elevated water temperatures the fungus may be inactivated or killed, as can occur in shallow water bodies at the peak of the summer months. Work undertaken by Lumanlan-Mayo *et al.*, (1997) monitoring outbreaks in rice-fish plots found that of the variables monitored, only water temperature played a significant role in EUS outbreaks. EUS did not occur during the 1995 dry season experiment even though water quality (excluding temperature) was similar to that during the 1993 and 1994 wet season experiments in which EUS outbreaks occurred. They suggested that the relatively high water temperatures (usually >35 ^{o}C) probably inhibited *Aphanomyces* activity.

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1.3 EUS prevention and control

At present there are no systemic treatments for use against EUS in fish. The very nature of the disease, fungal hyphae penetrating deep muscle and tissue, results in the physical protection of the pathogen from the external environment.

Research has therefore mainly targeted prophylactic and preventative treatments or measures to prevent the transmission of the disease, rather than treatment of affected fish. The control of EUS in wild fish populations, in open water bodies, is most likely impossible. All research efforts have been directed at management techniques and therapeutics for aquaculture, notably pond culture systems.

1.3.1 Preventative management techniques

Farm management techniques that are employed to control the incidence and spread of EUS are often diverse, specific to farm type, and species farmed. However there are preventative measures that are common to most pond systems that are used to exclude pathogens from the pond environment. Many are standard practices to reduce incidence of all disease, and therefore relevant to EUS.

It is likely that EUS is spread geographically, and persists locally, by affected or carrier fish, most probably in wild fish populations. The effort to exclude all wild fish from the farm site, and the collection of seed stock, and broodstock from disease free areas must be made.

Contaminated water and equipment would also result in the spread of the disease within and between individual farms, and sterilization of equipment must therefore become routine.

1.3.2 Control Treatments

Present treatments for EUS within Asian aquaculture range from compounds such as lime and salt for the sterilization of ponds to the topical treatment of diseased fish with crushed neem leaf and turmeric (Anon, 1994). There are a few commercially available treatments, such as malachite green, potassium permanganate, or CIFAX (a claimed EUS treatment marketed in India), however these are either potentially harmful to humans, very expensive, or have no confirmed efficacy.

Present snakehead farming practices in Thailand involve draining and sterilizing ponds prior to stocking, by the addition of lime (preferably slaked lime) and salt, spread evenly over the bottom. Water is then supplied to a depth of 10 cm for 7-10 days before filling to the normal depth and stocking with fish (Annex 2). When an outbreak of disease occurs fish are usually harvested, and ponds treated as above before restocking.

A common prophylactic measure in Asian aquaculture is the practice of liming ponds as a measure to combat disease in general, and improve water quality. Liming is an agricultural practice that has been adopted by fish culturists, and liming materials used in ponds are the same as those applied to agricultural soils. Compounds useful as liming materials contain either calcium or calcium and magnesium associated with an anionic radical that will neutralize acidity. The liming material most commonly used is agricultural limestone that is prepared by finely crushing limestone. This reduces the acidity of ponds to

improve the culture of fish species (survival, reproduction, and growth). Agricultural lime buffers the pH of the pond, however it has no sterilizing effect. Two other common liming materials are $Ca(OH)_2$ (calcium hydroxide, slaked lime) and CaO (calcium oxide, quicklime). These two compounds have been reported as having a therapeutic effect against pathogens, and the addition of quick lime (CaO) into fish ponds during EUS outbreaks appears to be effective in controlling losses in fish ponds (Chinabut, 1994). CaO has a cation exchange capacity, which may interact with mud or clay particles suspended and carried through the water column. Should *Aphanomyces invadans* have some connection with, or be supported by suspended particles, the addition of lime could cause the particles to flock out. Calcium hydroxide and calcium oxide however are unpleasant and dangerous to handle as they are highly caustic, and may also cause the water pH to rise to toxic levels to fish and inhibiting bacterial decomposition of organic matter.

There is often some confusion as to the actual composition of locally available liming materials. Various other materials, including ground seashells, flue dust, marl, and wood ashes, have been used for liming. In some developing countries, wood ash is abundant due to the wood fires used for cooking. Analysis of wood ash samples in some cases has been shown to have about 30-40% the value of agricultural limestone for use in ponds (Boyd, 1990).

An interesting method of disease management as practiced at one snakehead farm visited in Thailand (Farm owner: Mr. Chok Chai, Suphanburi province,

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1999, Annex 2), involved the deliberate increase in organic loading of the ponds. The water supply is cut off as soon as a disease outbreak occurs, and the algae allowed to bloom and then crash, presumably to kill pathogens in the water. The pond is then drained to half capacity, fish left to stir up the mud bottom, and a pulse of water is washed through to flush out organic matter. After the culture cycle the ponds are drained, and again salted and limed. This method would seem to go against all recommended preventative EUS measures, however disease has not been a problem for this farm in the last 4 years. It is also interesting to note that a study undertaken by Lilley (1992) assaying pond water for spores of saprolegniaceous fungi, found that in ponds with a high algal content, the spore density of aquatic fungus was 26 times lower than in similar ponds with little algal growth. This could possibly be a result of the organic matter and algae inhibiting fungal growth, competing for nutrients, or producing pH changes (through algal respiration and photo synthesis) unsuitable for fungal proliferation. If further work confirms field observations, this could prove a valuable, if slightly novel, control method.

1.4 Objectives

The objectives of this present study are threefold :

1. To attempt to develop a reproducible bath challenge system, to enable the study of causal factors contributing to EUS disease in snakehead.

2. To screen potential fungicides for effectiveness against the *Aphanomyces invadans* fungus (strain B99C) *in vitro*.

3. To use the developed bath challenge system to assess the efficacy of nominated fungicidal compounds at the tank trial level.

Fulfilling these three objectives would enable the study of EUS in snakehead to be undertaken without the use of injection of *A. invadans* spores to induce EUS, and therefore more closely imitate natural infection.

Novel compounds could be quickly screened for fungicidal activity, and then applied in tank trials, highlighting fungicides of potential for pond trials.

CHAPTER 2

2. METHODS

2.1 Maintenance of *Aphanomyces invadans* cultures

A pathogenic isolate of *A. invadans*, B99C, was isolated by J.H. Lilley and M.H. Khan from an EUS diseased fish (*Cirrhinus reba*) in the Mymensingh district, Bangladesh, 1999. The isolate was confirmed as *A. invadans* by its morphology, pathogenicity, growth, and reactivity to *A. invadans* specific PCR primers at AAHRI. The B99C isolate is maintained at AAHRI, in the institutes fungus culture collection.

The B99C isolate was used as the challenge strain throughout the investigation.

Cultures were stored on slopes of PG-1 agar (Annex 3) in universal tubes, with sterile light paraffin oil covering the entire slope, (as described by Smith and Onions (1994)). Cultures were recovered by removing the paraffin oil, and aseptically transferred to GPY agar plates. *A. invadans* cultures were maintained on GPY agar plates (Annex 3), incubated at 20° C, for a maximum of 7 days before being subcultured. Cultures growing longer than 7 days were discarded.

2.2 Inducing sporulation in *Aphanomyces invadans* cultures

Agar plugs 4mm in diameter were taken from the growing edge of the B99C fungal colonies. The plugs of mycelium were then placed in Petri dishes containing V8 broth (Annex 3) , and incubated for 4 days at 20 C. The resulting mycelial wads were then washed six times in distilled water, transferred to Petri dishes containing 20ml of autoclaved pond water (APW)(Annex 3), and incubated at 20 ^{o}C for 24 hrs. After 12 hours, the formation of achlyoid clusters of primary cysts and the release of motile secondary zoospores were apparent under the (compound) microscope.

To assess the feasibility of *in situ* sporulation in tanks, prior to experimentation, preliminary investigations involving cultures of B99C showed that sporulation occurred in: sterile distilled water, sterile APW, sterile MD (mineral distilled), and low alkalinity, low hardness water.

2.3 Experimental reproduction of EUS in snakehead: Bath Challenge.

2.3.1 Fish

A total of 600 wild caught, two month old, juvenile snakehead (*Channa striata*) were obtained from a commercial snakehead farm (Song Pee Nong District, Suphanburi Province), and held for a minimum quarantine period of 2 weeks in 150 litre glass tanks containing tapwater at $30^oC \pm 2^oC$, (at a stocking density of 40/tank). Prior to experimental challenge, fish were transferred to identical tanks in a temperature controlled room ($20^oC \pm 1$), where they were held for a minimum period of 1 week. Fish were fed with a commercial catfish diet, once per day. Feed was withdrawn 24 hours prior to experimentation.

2.3.2 Inoculation protocol

A total of 91 mycelial wads (7 per treatment tank) were grown up from agar plugs (as described above) and introduced into each treatment tank. 100 mycelial wads were grown in total, with a density of 10 wads per petri dish, in all cases.

Of the seven fungal wads per tank grown in V8 broth, three fungal wads were washed in distilled water, as described above, and placed within a plastic cage (made from a 15ml centrifuge tube, with holes and grooves burnt along its length) and placed directly in the tanks to sporulate *in situ*. The remaining four fungal wads per tank were washed in distilled water and left to sporulate overnight before being introduced into the cage with the other four mycelial

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wads. Zoospore counts of the *in vitro* sporulated wads were recorded, and the APW used for sporulation was pooled and divided equally amongst tanks to ensure the presence of an equal number of propagules in each tank.

Fungal wads remained within the tanks until day four, whereupon water was exchanged and fish were fed. Waste water was sterilized before discharge.

2.3.3 Exposure treatments

For the development of the bath challenge and investigation of water quality factors contributing to EUS, fish were stocked in 20 litre tanks, at a density of 10 fish per tank.

Water was not aerated at any time, and water changes occurred every two days during the 'acclimation' of the fish, and during the 'recovery' period, with 80% of the tapwater being replaced. Directly following treatment, 'exposure' water was completely replaced with tapwater in all cases. Tanks were blocked and randomised.

'Exposure' water samples consisted of (i) tap water, (ii) distilled water, and (iii) acidified distilled water (pH 5.0). Acidified water was achieved by the addition of 1.5gms/litre of sodium phosphate monobasic (NaH_2PO_4) to distilled water. All water samples were stored for two days at 20^0C prior to treatment. At this time water analysis was carried out using a Hach (Ames, Iowa,USA) water analysis test kit, and results were confirmed by independent analysis at the

National Inland Fisheries Institute (NIFI), Water Quality Laboratory, Department of Fisheries, Bangkok (Table 1).

In preliminary experiments the sublethal exposure period to acidified tap water was identified (Miles, unpublished), as the highest duration exposure to acidified water which allowed survival of all fish, without significant clinical abnormality, for at least 5 days post exposure in tap water. A water pH value of 5 was found to be acceptable to the fish, with no visible signs of distress, for at least 24 hours. This value is also comparable to the lower limit values recorded by Chinabut (1994) in her review of environmental factors in relation to EUS in Thailand. It is interesting to note that although a value of pH 5 seems a low level to maintain fish, Sammut (pers. comm.) and Callinan have observed wild populations of gudgeon (*Hypseleotris spp.*) surviving in acid sulphate water of pH 2.2, for 2-3 weeks, seemingly unaffected and acclimated.

Table 1. Water quality parameters measured , and confirmed values

	pH	Alkalinity (mg/l)[1]	Hardness (mg/l)[2]
Tap water	7.3	70	110
Distilled water	7.0	<1	<1
Acidified water	5.0	<1	<1

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[1] Hardness is a measurement of mineral ions in the water, the preponderance of which is Calcium and Magnesium. General hardness (GH) is usually expressed in terms of the amount of calcium carbonate ($CaCO_3$) present in solution. It is measured in degrees of German Hardness (^{o}dH) which can be convereted to ppm by multiplying by 17.

[2] Alkalinity is the measure of the amount s of carbonate ($CO_3^{=}$) and bicarbonate (HCO_3^{-}) ions. Alkalinity is related to the buffering capacity, or pH stability of the water. Test kits measure the alkalinity as carbonate hardness or KH often in degrees of German Hardness (^{o}dH), which can be converted to ppm by multiplying by 17.

2.3.4 Histological sampling

Fish were sampled throughout the duration of the experiment (30 days) whenever a dermal lesion appeared. Some lesions were allowed to progress to moderately advanced stages to provide information on granulomatous responses, and to examine possible recovery and healing responses of infected fish at this temperature. Moribund fish and any fish that were seen to be stressed as a result of infection were immediately sampled. Dead fish were sampled only if the period between death and sampling was known to be short (3-4 hours).

Fish were euthanased by striking the cranium, followed by decapitation.

Muscle samples were taken by cross-sectional cutting of 1cm thick sections, to include the lesion, and fixed immediately in cold 10% buffered formalin (10-15 times sample size volume). Samples were retained in formalin for a minimum of 48 hours. Samples were trimmed and then decalcified to aid sectioning, and then processed using an automatic tissue processor. Samples were impregnated with and embedded in paraffin wax. Samples were sectioned using a microtome to a thickness of 5 um. Sections were de-waxed, taken to water, and then stained either with haematoxylin and eosin to identify mycotic granulomas, or with Grocott's modified silver stain to reveal fungal hyphae (Annex 4), or a combination of the two, counterstaining with haematoxylin and eosin.

Sections were examined using a compound light microscope.

2.4 Assessment of potential fungicides against *Aphanomyces invadans*

The method outlined by Bailey (1983) for the screening of potential aquatic fungicides was used. This method involves using agar plugs containing fungal hyphae removed from the edge of actively growing colonies, and then challenged *in vitro* with an array of potential treatments and concentrations to provide an indication of *in vivo* activity. This approach enables many compounds to be screened simultaneously, in a relatively short space of time. Bailey recommends that the mean activity of each test should be carried out in triplicate for each compound and be compared with the activity obtained for malachite green. Theoretically, selected fungicides should control aquatic fungal growth for at least 48 hrs (Bailey, 1983).

Criteria for acceptance or rejection of candidate aquatic fungicide compounds (based on Bailey, 1983; Marking *et al*., 1994):

1. The activity of the candidate fungicide must be less than 100 mg l^1.

2. A candidate fungicide must show the desired level of activity in 1 h exposures, except candidates for pond treatments.

3. The efficacy of the fungicide must be reproducible in repetitive tests and be at least 50% that of malachite green after 48 h of incubation

4. The fungicide must be soluble in suitable carriers or capable of remaining in homogenous suspension to provide an effective contact time

5. The fungicide must kill (fungicidal action) or totally inhibit (fungistatic action) the growth of mycelia for at least 48 h after exposure.

6. There must be a satisfactory margin of safety between therapeutic and toxic concentrations.

2.4.1 Mycelium minimum inhibitory concentration (MIC) tests

Chemicals/fungicides were screened for activity against *A. invadans* mycelium using an adaptation of the method of Bailey (1983). Candidate chemicals were screened against distilled water and malachite green (1 ppm) as a positive control (Table 2). A range of concentrations were tested to evaluate the effective minimum inhibitory concentrations.

Four mls of each concentration of chemical were pipetted into different compartments of a multi-compartment 'Replidish' (Bibby Sterilin Ltd, Stone, Staffs, UK). Triplicate agar plugs were placed into the different fungicide solutions for 60 minutes exposure at each concentration. Agar plugs were then washed three times in separate replidish compartments containing 4 mls sterile distilled water over a period of 1 hour: first wash was 10 mins, second wash 20 mins, third wash 30 mins. The agar plugs were then blotted dry on sterile filter paper and placed on GP-agar medium in a three-sectional area of a petri dish. Cultures were then incubated at $20 \pm 1\ ^{\circ}C$, and examined under a binocular microscope for fungal growth after approximately 24, 48, 96, and 168 h. The results were recorded as negative (effective) if there was no fungal growth at each time interval tested, and positive if fungal growth was observed.

Table 2. Compounds tested for fungicidal activity against *A. invadans.*

Chemical / Extract	Current use and previous reported activity
D-limonene (Florida Chemical Co., Ltd., Winter Haven, Florida, USA)	D-Limonene is a biodegradable solvent occurring in nature as the main component of citrus peel oil. D-limonene can be used in its pure form, blended with other solvents & drying oils, or emulsified to make water soluble products. D-limonene has found a variety of applications, but most widely as a biodegradable solvent and degreaser.
E-Z-Mulse^{TM} (Florida Chemical Co., Ltd., Winter Haven, Florida, USA)	E-Z-Mulse^{TM} has recently been launched as a proprietary emulsifier. D-Limonene and E-Z-Mulse^{TM} readily combine with water to make stable cleaning formulations. E-Z-Mulse^{TM} is essentially non-hazardous, nearly odorless, biodegradable, and does not contain suspect nonyl phenol. E-Z-Mulse^{TM} is a single blend micro emulsifier, designed to work with naturally occurring organic solvents such as d-Limonene (derived from citrus), new synthetic solvents, and other terpenes.
Commercial Neem Extract (Office of Research and Development of Botanical Pesticides, Bangkok, Thailand)	Defatting procedure involving the addition of crushed seeds to hexane or pentane for 5 Hours, based on the method of Schroeder and Nakanishi (1987). Removal of solids (waste), by coarse filtration. Extraction based on the method used by Butterworth and Morgan (1971). Dilution of filtrate with 95% ethanol followed by filtration. Filtrate concentrated. Commercial extract contains 0.27% active ingredient (azadirachtin).
Neem Seed Extract	Seeds of *Azadirachta siamensis* crushed to a powder (seeds finely chopped using a blender). 50 g with 1 litre of sterile distilled water, stirred periodically and left overnight. Filtered with a linen cloth. Filtered through 0.45 um Whatman filter cassette. Filtrate used within 2 days - not stable, especially at higher temperatures.
Neem Leaf Extract	Fresh leaves of *Azadirachta siamensis* roughly chopped with a blender. 20g / litre sterile distilled water, stirred periodically and left overnight. Filtered with a linen cloth. Filtered through 0.45 um Whatman filter cassette. Filtrate used immediately.
Malachite Green	Used in fish aquaculture facilities worldwide to control or prevent freshwater fungal outbreaks. Use is restricted in many countries, often only licensed to treat fish eggs. Selection of aquatic fungicides frequently assessed against malachite green as the reference compound.

2.4.2 Zoospore production inhibitory assay

Chemicals were screened for zoospore production using a modification of the MIC test previously described in section 2.4.1. A range of concentrations was first tested for all chemicals. Distilled water and APW were used as controls.

The method for zoospore production developed by Lilley (1997, adapted from Willoughby and Roberts, 1994) using a V8 broth to grow up mycelial mats was used. Briefly, 4 mls of each chemical solution was diluted with APW and placed into Replidish compartments. After gently washing six times with distilled water, 3 agar plugs were placed with the selected chemicals. Cultures were then incubated at $20^{o}C$, and examined under a light microscope (40x magnification) for zoospore production after approximately 24 and 48 hours.

An effective concentration was recorded when no zoospores were produced. No attempt was made to enumerate zoospore population density in this test, however population density was marked subjectively (Table 7). The effective concentration of each chemical that gave total inhibition, or disruption, of zoospore production was used as a guide for further testing.

2.5 Assessment of potential fungicides – Tank trial bath challenge

The efficacy and maintenance of fungicidal properties displayed by various compounds was examined over time. This was attempted by the serial inoculation of tanks containing the test compounds, sampling of previously introduced fish, and the introduction of freshly abraded fish after each inoculation. The experiment was run over 15 days, with tanks inoculated, and freshly abraded fish introduced every five days (day 0, day 5, and day 10).

2.5.1 Fish

A total of 300 wild caught, two month old, juvenile snakeheads (*Channa striata*) were obtained from a commercial snakehead farm (Song Pee Nong District, Suphanburi Province), and held for a minimum quarantine period of 2 weeks in 150 litre glass tanks containing tapwater at $30^oC \pm 2^oC$, (at a stocking density of 40/tank). Prior to experimental challenge, fish were transferred to identical tanks in a temperature controlled room ($20^oC \pm 1$), where they were held for a minimum period of 1 week, with the addition of a 0.5% salt treatment to aid acclimation.

Fish were fed with a commercial catfish diet, once per day during acclimation. Feed was withdrawn 24 hours prior to experimentation, and fish were unfed for the duration of the experiment. Fish were euthanased by overdose of benzocaine.

2.5.2 Treatments

Treatment concentrations were chosen on the basis of their claimed or observed fungicidal activity (Table 3). No exchange of water occurred for the duration of the trial (15 days) once treatments had been added to tanks (day 0).

Treatments were duplicated in all cases, and tanks blocked and randomised.

Table 3. Compounds examined in tank trials to assess fungicidal effectiveness over 10 days

Treatment	Concentration	Comment
E-Z-Mulse™	10 ppm	Effective at disrupting 'normal' zoospore production as shown by zoospore production inhibitory assay.
Commercial neem seed extract	50 ppm	The only other potential treatment identified, disrupting zoospore production, as observed in zoospore inhibitory assay.
CIFAX	1ppm	A commercially available product claimed by Indian workers to be a curative and preventative treatment for EUS. 10 X recommended dose.
CaO	75 ppm	Addition of quick lime (CaO) into fish ponds during EUS outbreaks appears to be effective in controlling losses in fish ponds.
Control (tapwater)	-	Positive control.

2.5.3 Inoculation protocol

A total of 100 mycelial wads (10 per tank / inoculation) were grown up from agar plugs taken from the growing edge of fungal cultures (as described for experimental bath challenge) and introduced into each treatment tank. 300 mycelial wads were grown in total, with a density of 10 wads per petri dish, in all cases.

Of the ten fungal wads per tank grown in V8 broth, five fungal wads were washed and placed within a plastic cage (made from a 15ml centrifuge tube, with holes and grooves burnt along its length) and placed directly in the tanks to sporulate *in situ*. The remaining five fungal wads per tank were washed as described before and left to sporulate overnight in APW before being introduced into the cage with the other five mycelial wads. Zoospore counts of the *in vitro* sporulated wads were recorded, and the APW used for sporulation was pooled and divided equally amongst tanks to ensure the presence of an equal number of propagules in each tank.

Fugal wads remained within the tanks until day five, whereupon fish were sampled and tanks were re-inoculated and restocked.

CHAPTER 3

3. RESULTS

3.1Bath Challenge

3.1.1 Mycology

Propagules were known to be introduced into each of the treatment tanks from zoospore counts of the APW pool used to sporulate the 52 mycelial wads *in vitro*, and the subsequent aliquotting of the pool equally between treatment tanks.

Zoospore counts of 4.72×10^4 per ml, were recorded from the APW pool. 15 mls of APW from the pool was introduced into each 20 litre treatment tank, resulting in a theoretical concentration of at least 35 zoospores per litre. Although efforts to record actual numbers of zoospores were attempted, this proved unsuccessful, as did confirmation of the presence of zoospores using PCR techniques.

Efforts to re-isolate the fungus from muscle underlying putative EUS lesions on the fish at day 30 proved unsuccessful. Re-isolation of the fungus was also scheduled to be attempted in fish within the contaminated control tank (pH 5 distilled water), however all fish died prior to the isolation attempt, and were therefore too old for this to be successful.

3.1.2 Gross Pathology

During, and immediately following, exposure of fish to acidified distilled water, increased numbers of loose scales and free mucus were noted in each of the treatment tanks and control tank. Pale, irregular flecks, consistent with exfoliating epidermal cell sheets were seen in most, but not all, of the fish in these treatment groups. Varying degrees of haemhorraging were observed in fish exposed to both distilled water, and low pH distilled water treatments 1 and 2 days after exposure.

EUS associated lesions occurred in all tanks inoculated with *A. invadans*. Red haemorrhagic lesions occurred primarily on the flanks and tail region of infected individuals (Figure 3A & B). Lesions progressed from small (1-2 cm) dermal ulcers with associated haemorrhaging, to large necrotic ulcers, with fungal growth penetrating deep into the musculature, and in some cases resulting in contralateral lesions (Figure 3C). Lesions were occasionally present on the operculae of infected fish.
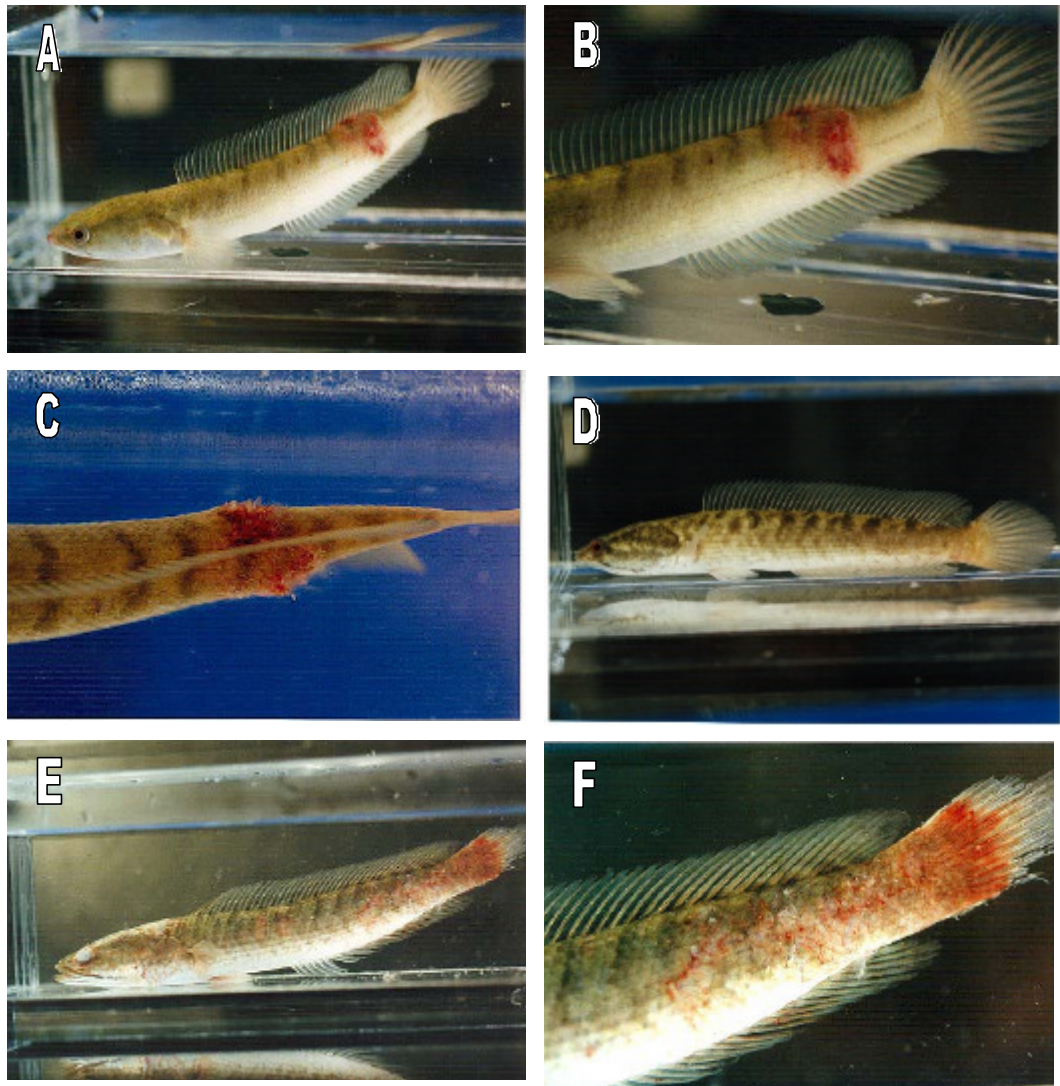
Figure 3. A & B, Snakehead exhibiting red haemorrhagic EUS lesion on tail (Day 12, *A. invadans* and distilled water). **C,** Contralateral lesion on tail showing deep invasion of fungus through musculature (Day 12, *A. invadans* and distilled water). **D,** Healthy control fish (Day 12, Tapwater). **E & F,** Lesions from bacterial infection (recently transported fish , held at 30 ^{o}C, low stocking density).

Lesions were also associated with *Achlya* infection in a number of tanks, especially those fish exposed to acidified distilled water (Figure 4). Fungal tufts were often heavy, and the infection rapid. Of note, is the increased incidence of *Achlya* infection covering the nares in those fish exposed to acidified distilled water.

Distilled water and acidified distilled water tanks inoculated with *A. invadans* displayed EUS-like lesions earlier (day 7-8) than fish exposed to fungus and tapwater (day 12-14). In surviving or sampled fish, no evidence was seen to indicate that lesions of any kind were healing, or had healed.

No EUS-like lesions were observed in the tapwater, or distilled water control tanks. Fish held in the pH5 distilled water control tank however, began to show EUS-like lesions from day 16 onwards, and contamination from an unknown source was suspected. Lesions were also associated with an opportunistic *Achlya* infection, and histological samples showed an intramuscular fungal infection consistent with *A. invadans*. Efforts to isolate fungus from infected tissue and extract DNA for PCR technique characterisation to positively identify the fungus were unsuccessful.
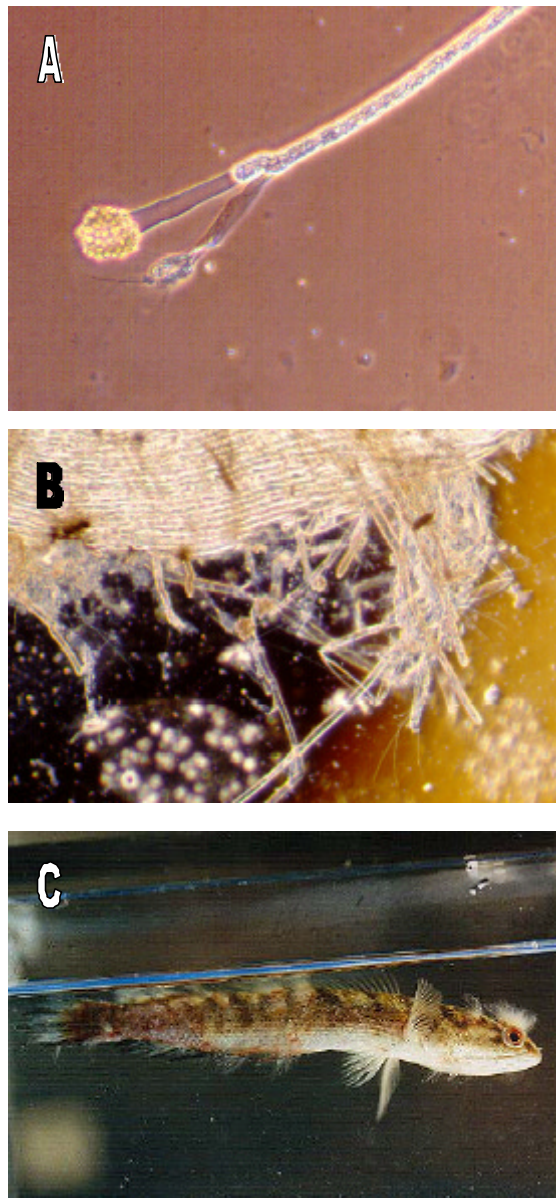
Figure 4. A, Photograph of *Achlya* species of saprophytic fungi, showing double row of primary zoospores contained within hyphae, and branching of hypha below the basal septum showing sympodial zoosporangial renewal (X 100 magnification). **B,** Fungal hyphae visible on an scale sampled from an *Achlya* infected fish (X 40 magnification). **C,** EUS affected fish showing opportunistic *Achlya* infection (Day 15, exposed to acidified distilled water).

3.1.3 Behavioural observations

Snakeheads exhibit despotic hierarchies, with a single individual, the despot, dominant over all other individuals, while subordinate animals have approximately equal ranks. In captive snakehead it was frequently observed (especially amongst the larger individuals) that a single snakehead would monopolize more than 80% of the tank, with the remaining fish massed together in continual contact. Territorial claim would almost always be the bottom of the tank.

Aggressive behaviour was observed as lateral displays with two fish swimming in place with fins spread, orientated anti-parallel. As an interaction escalates, fish begin body beating, a vigorous swimming in place that pushes water at an opponent and that may indicate relative strengths of the combatants. Fish then 'carousel', swimming in tight circles around one another, which can lead to biting of caudal fins or chasing. This aggressive behaviour, often resulting in severe biting by some fish, led to dermal damage, particularly of the tail region. This damage often led to bacterial infections, and would therefore suggest a route of entry for pathogens, including *A. invadans*, by compromising epidermal defenses and mucosal immunity. This in turn may lead to the development of lesions, and may explain the higher prevalence of lesions on the tail region, and explain non-EUS lesions observed on control fish.

3.1.4 Histopathology

Positive identification of EUS was confirmed by the presence of fungal hyphae, morphologically consistent with *A. invadans*, invading the musculature directly underlying an area of dermal ulceration (Figure 5 A-D), with extensive necrotising granulomatous dermatitis and myositis (Figure 6). Varying degrees of macrophage response and inflammatory response were observed in all treatments. Fungal hyphae could be seen invading the gonads (Fig 5 E) of some individuals. An average hyphal width of 18 um in positively identified tissue, is consistent with values obtained by Willoughby *et al.* (1995).

A number of individuals, most notably in the fungus and tapwater exposed fish, showed fungal hyphae present in the dermis (Fig 5 F). This was morphologically consistent with *A. invadans* and hyphae present intramuscularly in positively identified samples. Due to the case definition of EUS stating an invasive infection and granulomatous response, these samples could not be recorded as positive. If however, the fungus was indeed established in the dermis, this condition may have eventually led to an identifiable EUS infection.

Samples were recorded as 0 for no fungal involvement within tissues, 1 if fungus morphologically consistent with *A. invadans* was observed within the dermis of the fish, and 2 if an EUS lesion and invasive fungus morphologically consistent with *A. invadans* was present (Summary Table 4, comprehensive results Annex 5).
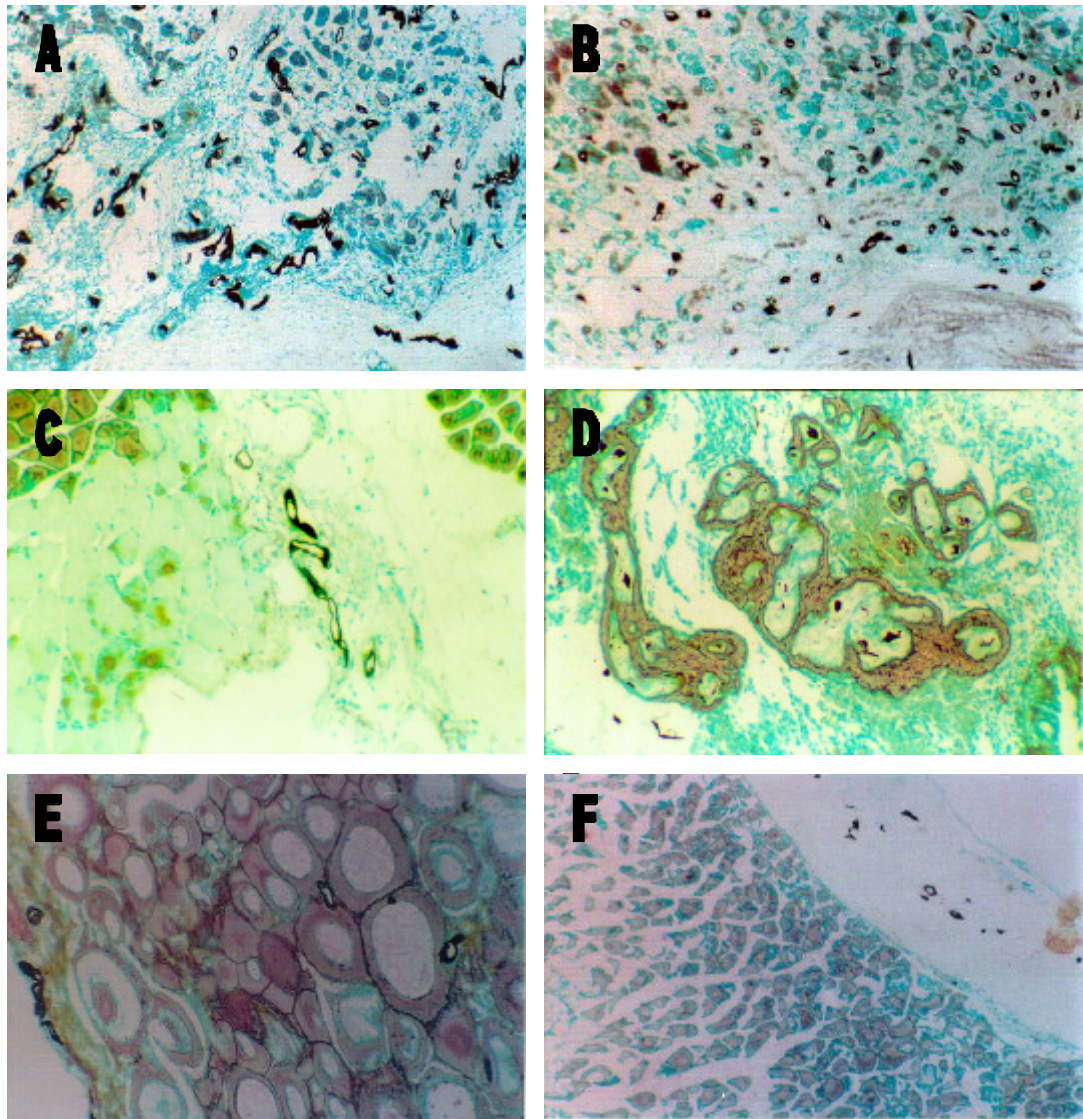
Figure 5. A – C, Sections showing invasive *A. invadans* hyphae penetrating deep into the musculature (Grocott's silver stain, X100, x100, X200 magnification respectively). **D,** Section of musculature showing fungal hyphae encapsulated by dense fibrous tissue. (Grocott's silver stain, X 100 magnification). **E,** Fungal hyphae seen penetrating gonadal tissue (Grocott's silver stain, X 200 magnification). **F,** Fungal hyphae present within the dermis, morphologically consistent with *A. invadans*. (Grocott's silver stain, X 100 magnification).
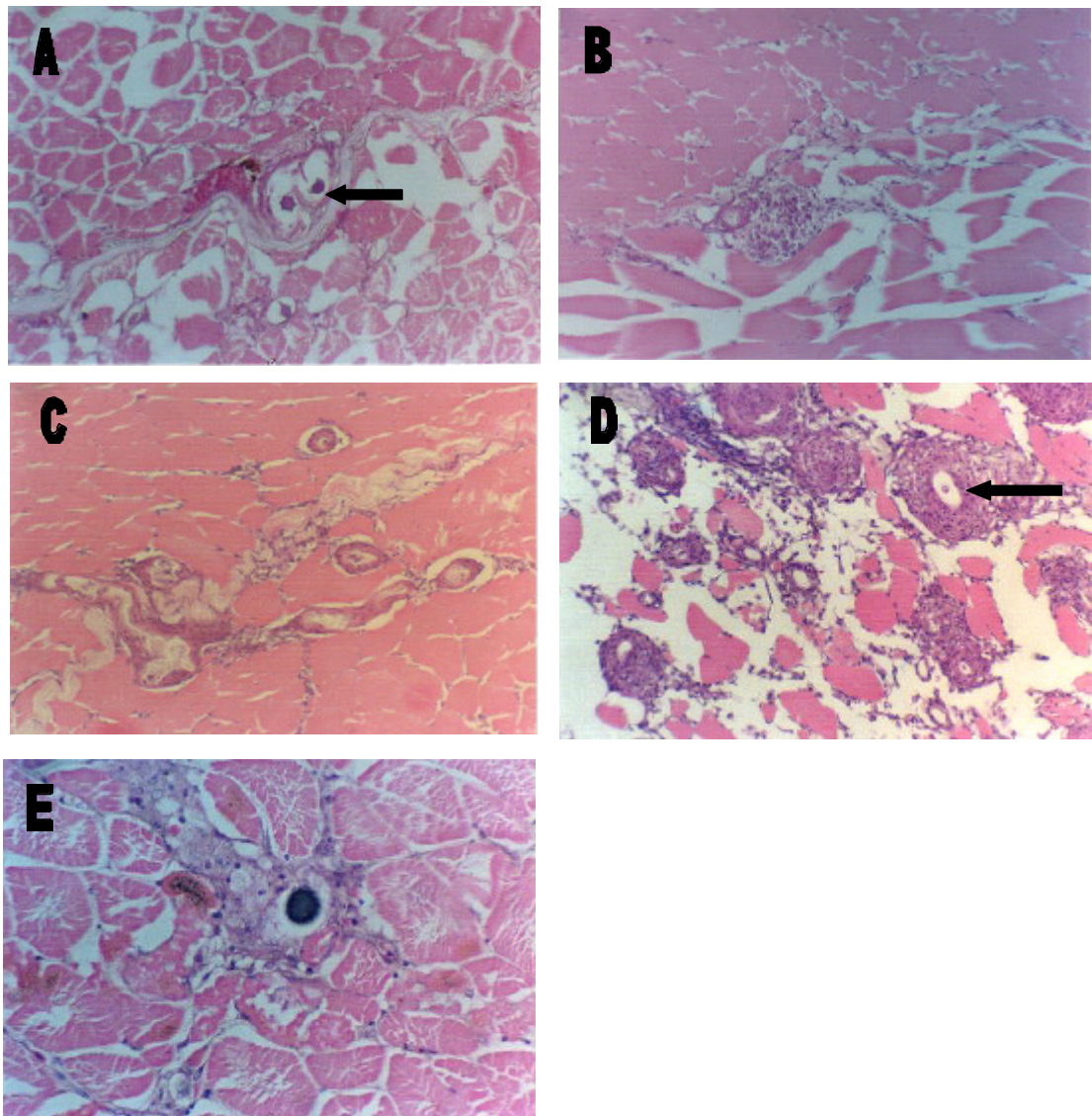
Figure 6. A, Encapsulated fungus (arrowed) with limited inflammatory response (H & E, X 200). **B,** Well encapsulated fungus, good granulomatous response, and increased inflammatory response. (H & E, X 100). **C,** Fungal hyphae emerging from enveloping granuloma. (H & E, X 200). **D,** Granulomas containing dead fungal hyphae (arrowed) are embedded in dense fibrous tissue. (H & E, X 100). **E,** Granuloma and associated inflammatory response. (Grocott's silver stain and H & E counterstain, X 400)

Table 4. Summary results of bath challenge experiment, showing percentage of EUS infected fish, and day of first histologically confirmed infection within treatment groups.

Treatment	Number sampled	Number positive (as 2)	Number negative (as 0 &1)	% positive	Total % positive	Day of 1st confirmed result
A invadans + Tapwater	9	4	5	44.4%	42.4%	16
	9	6	3	66.6%		15
	9	2	7	22.2%		14
	6	2	4	33.3%		15
A invadans + Distilled water	10	7	3	70%	50%	7
	8	2	6	25%		7
	11	4	7	36.4%		11
	9	6	3	66.6%		12
A invadans + pH 5 Distilled water	10	7	3	70%	72.5%	8
	11	9	2	81.8%		14
	11	8	3	72.7%		13
	9	6	3	66.6%		14
A invadans + Tapwater + Scrape	8	5	3	62.5%	62.5%	16
Tapwater	9	0	9	0%	0	0
Distilled water	9	0	9	0%	0	0
pH 5 Distilled water	7	4	3	57.1%	57.1%	16

3.1.5 Bath challenge data analysis

The incidence of EUS within groups was examined for evidence of significant differences between treatments. Data was analysed using one-way analysis of variance (ANOVA). The P-value of the F-test is greater than 0.1, which results in treatment groups not being statistically different from one another at the 90% confidence level (Table 5).

The method used to discriminate among means of the groups is Tukey's honestly significant difference (HSD) procedure (Figure 7). There are no statistically significant differences between any pair of the means at a 90% confidence level.

Table 5. ANOVA Table for incidence of EUS within groups compared to exposure treatment.

Source	Sum of Squares	Mean Square	F-Ratio	P-Value
Between groups	1583.45	791.723	2.47	0.1395
Within Groups	2883.29	320.366		

Figure 7. Comparison of the incidence of EUS against exposure treatments
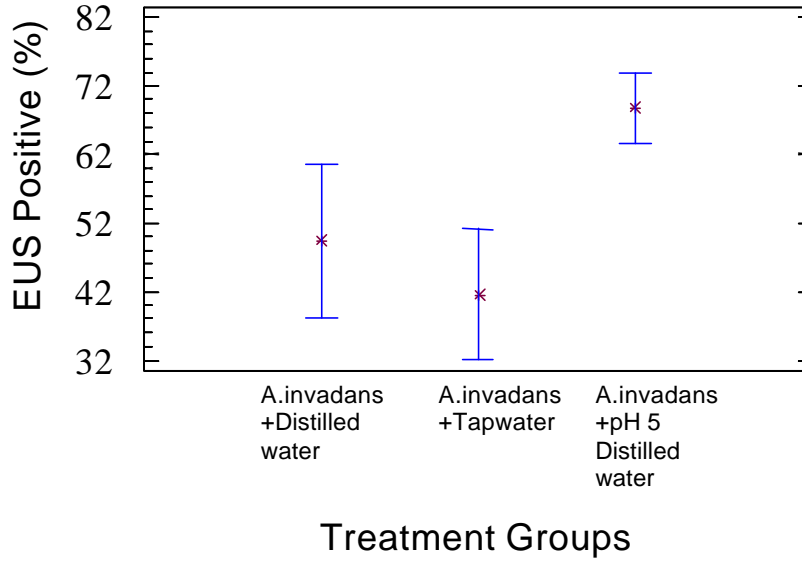
3.2 Assessment of preventative treatments

3.2.1 Mycelium minimum inhibitory concentration (MIC) tests

Of the seven chemical compounds screened for fungicidal activity against *A. invadans*, only three compounds, malachite green (control), E-Z-Mulse[TM] and to a lesser degree, commercial neem extract, showed any sustained activity as a fungicide (Summary results Table 6, comprehensive results Annex 6).

D-limonene was insoluble in water, resulting in minimal contact between compound and mycelium, and consequently found to be ineffective at any concentration.

The d-limonene E-Z-Mulse[TM] emulsion did show fungicidal activity, however the degree of activity was directly related to the concentration of E-Z-Mulse[TM] within the formulation. Results would therefore suggest activity of E-Z-Mulse[TM] is the regulating factor in activity.

E-Z-Mulse[TM] showed good fungicidal activity at relatively low concentrations when compared to the other compounds tested, and the activity of E-Z-Mulse[TM] was sustained for longer than the control, remaining a viable fungicide for more than 96 hours.

Commercial neem extract did show ability to inhibit mycelial growth for more than 48 hours, however effectiveness was lost at 72 hours.

Both the neem seed and neem leaf preparations were shown to be ineffective at any concentration tested, and if anything, the neem leaf preparation could

be seen to actually increase growth of mycelium (width of colonies) when compared to the negative control (no chemical treatment).

Malachite green, positive control and fungicide standard, showed fungicidal activity for at least 48 hours at 1ppm, conforming to Bailey's criteria for fungicidal compounds.

Table 6. Summary results of Mycelium Minimum Inhibitory Concentration (MIC) test

Compound	Minimum concentration to inhibit mycelium growth for 48 hrs	Comments
D-limonene	>10000 ppm	Insoluble in water, therefore ineffective
D-limonene + E-Z-Mulse^{TM}	50 ppm	Effective for at least 96 hours
E-Z-Mulse^{TM}	25 ppm	Effective for at least 96 hours
Commercial Neem Extract	1500 ppm	Ineffective at 72 hours
Neem Seed Extract	>75 000 ppm	Ineffective
Neem Leaf Extract	>75 000 ppm	Ineffective
Malachite Green (control)	1 ppm	Ineffective at 72 hours

3.2.2 Zoospore production inhibitory assay

The results of the inhibitory assay show that none of the compounds tested totally inhibited zoospore production. Of the compounds tested, only the d-limonene/E-Z-Mulse^{TM} formulation, E-Z-Mulse^{TM} and commercial neem extract resulted in any alteration or disruption to normal zoospore production in *A. invadans* (Summary results Table 7, comprehensive results Annex 7). Both the d-limonene/E-Z-Mulse^{TM} formulation and commercial neem extract resulted in immediate encystment of secondary zoospores upon release. Not all primary zoospores were released.

E-Z-Mulse^{TM} treatment showed similar results, however fewer cysts were observed. Unreleased primary zoospores then began to germinate while still attached as a cluster, producing a highly branched, thin and wispy hyphae.

Neem seed and neem leaf preparations again resulted in a seemingly enhanced fungus activity and an increased production of zoospores.

Table 7. Summary results of zoospore production inhibitory assay.

Compound	Concentration (ppm)	Result after 24 hours	Result after 48 hours	Comments
D-limonene	100ppm	++	++	Insoluble, therefore ineffective
D-limonene + E-Z-Mulse^{TM}	100 ppm	0	0	Few cysts
E-Z-Mulse^{TM}	10 ppm	0	00	Highly branched, very thin sporangia
Commercial neem extract	50 ppm	0	0	Very few cysts
Neem seed extract	500 ppm	++	++	Many cysts
Neem leaf extract	10000 ppm	+++	+++	Many cysts

-     = no clusters, no zoospores produced

+   = clusters, no zoospores

++   = few zoospores

+++ = many zoospores

O   = clusters present, zoospores immediately encyst.

OO = clusters present, germlings produced from zoospores still attached to

      cluster

3.3 Treatment efficacy bath challenge investigation

3.3.1 Mycology

Each time tanks were inoculated, zoospore counts of the APW pool used to sporulate the 50 mycelial wads *in vitro*, were taken. Propagule counts were then estimated and introduced into each tank by aliquotting of the pool equally between tanks, as described previously.

Zoospore counts of 1.4×10^3 per ml, 1.1×10^3 per ml, and 5.6×10^2 respectively, were recorded from the APW pools from successive sporulations, resulting in theoretical concentrations of at least 20, 16, and 8 zoospores per litre. Again efforts to record actual numbers of zoospores proved unsuccessful, as did confirmation of zoospore infection using PCR techniques.

3.3.2 Gross Pathology

No EUS-like lesions were observed on any of the fish at any time. Two individuals succumbed to bacterial infections, but this was thought to have been the result of handling stress in transfer between tanks, and associated loss of scales from netting.

Visually, areas of abraded skin appeared to start healing 48 hours after the initial insult.

3.3.3 Behavioural observations

The reduced stocking density of fish greatly reduced the severity of chasing and biting of other individuals in the groups by the dominant individuals. Although hierarchies were established, and lateral displays were observed, fish appeared far less aggressive than those at higher stocking densities. As before, territorial claim would almost always be the bottom of the tank.

3.3.4 Histopathology

Positive identification of *A. invadans* infection was confirmed in only four out of the twenty individuals within the two positive control tanks among the first group of exposed fish. Fungus was observed within the dermis, underlying the area of initial abrasion and resulting epidermal repair.

Of the samples examined from the treatment groups inoculated with *A. invadans*, no fungal growth within tissues was observed.

Due to only four out of the twenty samples within the control groups revealing a positive result, examination of subsequent samples was abandoned. Further examination of samples will be undertaken at a later date.

CHAPTER 4

4. Discussion

Bath challenge

From the statistical analyses performed on treatment groups and incidence of EUS it can be seen that there are no differences between tapwater and low pH distilled water, between distilled water and tapwater, and distilled water and low pH distilled water. This could be due to the distilled water increasing likelihood of infection, rather than exposure to low pH. Although no differences can be observed between tapwater and the distilled water groups, tapwater of this alkalinity and hardness is still regarded as soft (alkalinity 70 mg/litre, hardness 110 mg/litre).

The results of the bath challenge experiment to assess EUS causal factors would indicate that both acidified water of low alkalinity, and low hardness and neutral water of low alkalinity, and low hardness increases the susceptibility of fish to EUS. This result supports findings by Callinan (unpublished), and the hypothesis that sublethal acidified water after a rainfall event increases the susceptibility of fish to EUS.

The distilled water treatments would have the effect of increasing the net loss of ions across the skin, particularly sodium and chloride, due to osmotic pressure. Exposure to distilled water would also result in loss of magnesium and calcium ions, important in membrane integrity and skin repair.

50

The responses of Amazonian fish to low pH in exceptionally pure water (Wood *et al.*, 1997) showed that some species exhibited a marked increase in Cl^- and Na^+ efflux modified by Ca^{2+}, with an associated stress response. Heisler (1996) also showed that chloride efflux of the integument depended largely on environmental Ca^{2+}, but was also affected by the extracellular bicarbonate concentration. Loss of these ions would also result in an impaired ability to transport O_2.

Stress in fish often results in pronounced disturbances in hydromineral balance, related to the aquatic environment and changes in epithelia covering the skin and gills. Wendelaar Bonga *et al.*, (1997) observed changes in epithelium resulting in an increase in the passive movement of water in stressed fish. There was associated swelling of intercellular spaces which were penetrated by different types of leucocytes. The increased permeability to water and ions, and possibly also to antigenic material and pathogens, may be caused by high circulating levels of catecholamines. They also noted that the incidence of cellular degeneration (by necrosis as well as apoptosis) was observed in respiratory cells and pavement cells, independent of whether the stressor was toxic (heavy metals, acid water with aluminium) or non-toxic (isolation, a submissive position in social hierarchy, or confinement).

Although there is an argument for infection without mechanical damage as outlined by increased efflux of ions into the surrounding environment,

behavioural observations cannot be ignored, and the aggressive territorial displays and increased incidence of biting would have resulted in mechanical damage and a compromised dermis.

The contamination of the acidified distilled water control tank in the bath challenge investigation most possibly occurred as a result of the accidental introduction of contaminated nets or hoses used to sample and drain infected tanks into the control tank. Although separate nets and hoses were used between infected and control tanks, this seems to be the most likely explanation. This further highlights the importance of the sterilization of infected equipment to control the spread of EUS between locales, as discussed before.

4.2 *In vitro* screening of potential fungicides

The mycelium MIC tests proved very useful in identifying potential fungicides against *A. invadans*. Of the compounds tested, E-Z-Mulse™ and the commercial neem extract showed the most promise for further investigation, clearly disrupting normal growth. It must be noted however that the method developed by Bailey (1983) to assess potential fungicidal compounds does indeed give a good indication of activity, but was developed to study the action of fungicides against surface saprophytes. In using the method to explore fungicides to target *A. invadans*, results should be treated with caution. The *A. invadans* mycelium invades body tissues and is subsequently shielded from the activity of an environmental or topical therapeutic (which this test was initially designed to assess). Bailey's method is extremely useful, and should be seen as a method on which to initially gauge the effectiveness of compounds against *A. invadans*. When assessing potential fungicides against *A. invadans*, unless the effective compounds could be used as a systemic treatment, this method as the sole method of analysis would be inappropriate. To assess compounds intended as an environmental treatment or preventative treatment, targeting of an alternative stage of the life cycle is needed.

The infective secondary zoospore is an obvious target when considering chemical control. Due to the movements of the zoospores within the water column, and consequent exposure to the aquatic environment, directed treatment is possible. either as a prophylactic effort, as a treatment of supply water, or to treat pond water once an outbreak is under way to arrest the

spread of disease. Of the compounds tested in the zoospore production inhibitory assay, none totally inhibited zoospore production. It was found that treatments that were effective against mycelial growth also had some effect against the secondary zoospore. Again E-Z-Mulse^{TM} (at 10 ppm)and the commercial neem extract (at 50 ppm) disrupted the normal growth and life cycle, and were therefore the two chosen compounds to further investigate in the fungicide treatment tank trials. Chemical concentrations required to immobilise zoospores were found to be considerably lower than those required to inhibit mycelial growth.

Although the E-Z-Mulse^{TM} and d-limonene combination did have some effect at 100 ppm, it was assumed that the effect observed was due to the E-Z-Mulse^{TM}, rather than the combination of the two. D-limonene, neem leaf extract and neem seed extract proved to be ineffective against the zoospores at all concentrations tested.

4.3 Assessment of potential fungicides - Tank trial bath challenge

Preliminary results from histological examination indicate that the bath challenge protocol used to test the effectiveness of potential fungicides, was unable to induce sufficient infection to enable successful screening of these fungicides. Under these experimental conditions, either the time period required for abraded snakehead fish to become infected by *A. invadans* must be longer than the five days used here, or epidermal recovery occurs before the secondary zoospores have the opportunity to colonize and grow in the exposed dermis. Previous work by Callinan and Sammutt (unpublished) clearly showed infection by *A. invadans* when skin is abraded, however they also noted recovery of exposed dermis 12 h post exposure, and advanced repair 24 h post exposure in these groups, although the epidermis often remained attenuated.

Another factor to consider is the growth and consequent performance of *A. invadans* held at this temperature. Although the fungus is still pathogenic at this temperature, the rate of growth is reduced (Chinabut *et al*, 1995., Lilley, 1997). Reduction in growth and activity of infecting zoospore, coupled with a sufficiently active, although retarded, snakehead immune response, may be sufficient in arresting infection at this temperature and time period. Future work should possibly extend the time period snakeheads are exposed to the fungus, or kept after the exposure to the fungus, however using the above protocol

would cause problems with accumulating organic matter and waste in the tanks, possibly to toxic levels.

 Although the tank challenge was unsuccessful in producing a positive EUS infection, it is valuable to note that E-Z-Mulse^{TM} and commercial neem seed extract at 10 ppm and 50 ppm respectively, appear to have no toxic effect to snakehead fish over the period of challenge (5 days).

E-Z-Mulse^{TM} is a recently launched product, and to our knowledge, untested on fish. The product has been marketed as a safe alternative to other harmful nonylphenol products which have confirmed estrogenic activity in many fish species (Burkhardt-Holm *et al.*,1997). Given that many surfactants tested seem to show aggressive fungicidal activity against *A. invadans* (Taukhid, 1999) this product could be a promising candidate treatment against the water borne *A. invadans* fungus.

The commercial neem extract at this concentration appeared to have no toxic effects on the snakehead. This is in marked contrast to recent findings showing highly toxic effect of neem on some species (Rungratchatchawal,1999), with calculated 96 hour LC_{50} (median lethal concentration) values of 0.185 mg/l for silver barb (*Barbodes gonionotus*) and 0.99 mg/l for striped catfish (*Pangasianodon hypopthalamus*), with safe concentrations being calculated as 0.00925 mg/l and 0.00495 mg/l respectively. Snakehead fish in this investigation were exposed to levels of 0.135 mg/l of active azadirachtin (50 mg/l crude extract) with no mortality. This would suggest that snakehead are seemingly resistant to the toxic effects of neem at these levels. However even

if neem is non-toxic to snakehead at this concentration, if the extract were allowed to enter the water system, other fish species and aquatic fauna may be affected. Even though neem extract is used in Vietnam against parasites, neem extract is used as a piscicide in India, supporting these recent findings, and consequently may find a severely limited application in treating fish disease, if at all.

4.4 GENERAL CONCLUSION

The bath challenge system developed shows that EUS can be induced in snakehead without the injection of *A. invadans* spores. This will enable the study of the causal factors of EUS in this species to be more fully explored in future.

The screening of potential; fungicides using the adapted method devised by Bailey (1983) to investigate fungicidal action on alternative stages of *Aphanomyces invadans* life cycle, appears to be effective at highlighting potentially active compounds.

The combination of the developed bath challenge and screened fungicides at the tank trial level, to assess efficacy of treatments, can be seen to be a valid method, however the length of time fish are kept after exposure and prior to sampling at this temperature will need to be assessed. Although not all samples in this section of the investigation were examined due to the initial failure of a sufficient infection in control tank groups, further histology of these and subsequent samples are being examined. These results will hopefully compliment work undertaken by the Department for International Development (UK) towards the investigation of the treatment and control of EUS at the pond level.

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Annex 1

Species naturally susceptible to EUS as indicated by the presence of typical mycotic granulomas in histological section or isolation of pathogenic *Aphanomyces* from muscle or internal organs. (adapted from Lilley, 1998)

Latin name (common name)

Acanthopagrus australis (yellowfin bream)

Anabas testudineus (snakeskin gourami)

Bidyanus bidyanus (silver perch)

Carassius auratus (crucian carp)

Carassius carassius auratus (gold fish)

Channa maculata (=Ophicephalus maculatus) (Formosan snakehead)

Channa marulia (=Ophicephalus marulius) (river murrel - India)

Channa micropeltes (=Ophicephalus micropeltes) (red snakehead)

Channa pleurophthalmus (=Ophicephalus pleurophthalmus) (snakehead)

Channa punctata (=Ophicephalus punctatus) (mud murrel - India)

Channa sp. *(=Ophicephalus* sp.) (snakehead)

Channa striata (=Ophicephalus striatus) (striped snakehead)

Catla catla (catla)

Cirrhina mrigala (mrigal)

Clarias batrachus (walking catfish)

Colisa lalia (dwarf gourami)

Epinephelus sp. (grouper)

Esomus sp. (flying barb)

Etroplus sp. (chromide)

Fluta alba (swamp eel)

Glossogobius giurus (bar-eyed goby)

Glossogobius sp. (goby)

Heteropneustes fossilis (stinging catfish)

Johnius sp. (croaker fish)

Labeo rohita (rohu)

Lepomis macrochirus (bluegill)

Liza diadema (mullet)

Macquaria ambigua (golden perch)

Mastacembalus armatus (armed spiny eel)

Mastacembalus pancalus (guchi - Bangladeshi)

Mugil cephalus (grey mullet)

Mugil sp. (mullet)

Mystus sp. (catfish)

Notopterus notopterus (grey featherback)

Osphronemus goramy (pla raet - Thai)

Oxyeleotris marmoratus (sand goby)

Oxyeleotris sp (gudgeon)

Platycephalus fuscus (dusky flathead)

Platycephalus sp. (flathead)

Plecoglossus altivelis (ayu)

Psettodes sp. (spiny turbot)

Puntius gonionotus (silver barb)

Puntius sophore (punti - Bangladeshi)

Puntius sp (puntius)

Rohtee sp (keti - Bangladeshi)

Scatophagus argus (spotted scat)

Scatophagus sp. (scat)

Sillago ciliata (sand whiting)

Sillago sp. (sillago)

Terapon sp. (therapon)

Trichogaster pectoralis (snakeskin gourami)

Trichogaster trichopterus (3-spot gourami)

Tridentiger obscurus obscurus (Japanese trident goby)

Upeneus bensai (goatfish)

Valamugil sp. (mullet)

Wallago attu (wallago)

Xenentodon cancila (round-tailed garfish)

Snakehead farming practices in Thailand

Snakeheads, belonging to the family Channidae, are highly regarded food fish in South and Southeast Asian countries. Their ability to breath atmospheric oxygen makes it possible to keep them alive for long periods of time, provided they are kept moist. As a result of this, snakehead (pla shon) can command a high price in the live fish markets. Besides the high-quality flavour and texture of their flesh, they are also regarded as beneficial in the diet for invalids and recuperating patients. In Thailand snakeheads are an important wild fisheries species in poorer communities, and in the Central provinces a commercially important semi-intensive culture species.

In Thailand the main culture species for food fish is *Channa striata* (695 farms, producing 5910 tonnes) however there is also a sizeable production of *Channa micropeltes* (182 farms,762 tonnes), with the fry also being sold as an aquarium fish. (Production figures from; Freshwater Fish Production 1993, Department of Fisheries, Fisheries Statistics Sub-Division, No.1/1993). Both species grow to considerable size (1-1.2 m total length) with preferred market size between 600 and 1000 g, which they attain in 7-8 months.

The most common system of snakehead culture in Thailand is in earthen ponds ranging in size from 800 m^2 to 1600 m^2 (1 rai) and 1 to 2 metres in depth. Here more intensive rearing is practiced, a continuous flow of water or frequent exchange of water is maintained.
Being highly predaceous and cannabalistic, snakeheads are generally raised in monoculture using, as far as possible, stock of the same size. In Thailand
fry are collected from the wild about 12 days after hatching. The fry exhibit some schooling behaviour at this time and are attracted to shelter in brush refuges (lom kram) set up along the banks of water bodies. The fry are then

harvested by surrounding the refuge with a seine net, and removing the twigs and branches from within. Fry are collected at night, and not during periods when water levels are high.

Fry are collected from March to October, and then grown out (for 4 or more months) in nursery ponds or tanks, to fingerling size (pla roon). Prior to stocking, ponds are drained and treated using lime and salt, flushed, and then stocked in (July and August) for grow out to market size in (April/May) the following year.

Fish are fed two to three times daily on a mixture of trash fish and rice bran in the ration of 3:1, and this enables the fish to attain market size (600-1000 g) in 7-8 months. A feed conversion ratio (FCR) of 6-7:1 is achieved when using this feeding regime, and although this is not as low as can be achieved using commercial catfish pelleted feed, prices are 5-7 baht per kilo (depending on season) and 30 baht per kilo respectively. Some farms remove feed after an hour, if uneaten (to reduce water quality spoilage) and this is then added to separate catfish ponds (*Pangasius sutchi*). Feeding is also reduced during periods of heavy rainfall, as fish are more stressed during this time.

A proportion of the fish are dried, but the majority is delivered live to market. Fresh snakehead currently fetch 95 Baht per kilo at market (40 Baht = $1).Dead fish do not command such a high price, usually fetching 30-40% less than the live product. Damaged or diseased fish are usually fermented (a consumed food item).
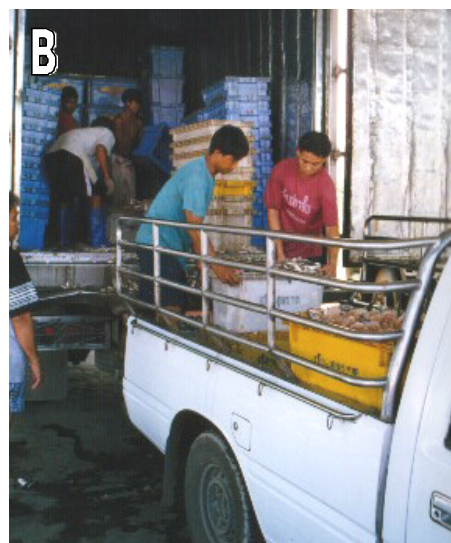
Figure 8. A, A typical snakehead pond, Thailand, 800 m^2, 2 metres deep, constant water flow. **B,** Delivery of fresh trash fish for feed preparation. **C,** Feed processing, mixing trash fish and rice bran in ratio of 3:1 respectively. **D,** Mechanical mixing of feed constituents.

Figure 8 continued. **E,** Fish fed 2-3 times daily to attain market size in 7-8 months. Note semi-submerged feeding platforms to reduce feed waste.   **F,** Live transport of fish to market. **G,** Dried fish for sale at a local market. An effective method of storing fish at tropical temperatures. **H,** Snakehead are highly prized food fish, with every part of the fish being used.

Annex 3

Formulae for media:

GP (glucose-peptone) broth

3 g/l	Glucose (BDH)
1g/l	Peptone (bacteriological, Oxoid)
0.128 g/l	$MgSO_4.7H_2O$ (Hopkins & Williams, Chadwell Heath, Essex)
0.014 g/l	KH_2PO_4 (BDH)
29.3 mg/l	$CaCl_2.2H_2O$ (Sigma)
2.4 mg/l	$FeCl_3.6H_2O$ (Sigma)
1.8 mg/l	$MnCl_2.4H_2O$ (BDH)
3.9 mg/l	$CuSO_4.5H_2O$ (BDH)
0.4 mg/l	$ZnSO_4.7H_2O$ (BDH)

GPY (glucose-peptone-yeast) broth

As GP broth with:

0.5 g/l   Yeast (BDH)

GP agar

As GP broth with:

12 g/l   Technical agar (Oxoid No.3)

GPY agar

As GPY broth with:

12 g/l   Technical agar (Oxoid No.3)

GP-PenOx broth

Prepare GP agar and after autoclaving and cooling to 50 ^{o}C add

100 mls   Penicillin-K (Sigma)

10ug/ml   Oxolinic acid (Sigma)

GP-PenStrep agar

Prepare GP agar and after autoclaving and cooling to 50°C add:

100 units/ml   Penicillin-K (Sigma)

10ug/ml   Streptomycin sulphate (Sigma)

V8 broth

5%   V8 juice (Campbell Grocery Products Ltd.)

0.2% $CaCO_3$

Centrifuge at 3000 rpm for 15 mins

Adjust pH to 6.1

APW (autoclaved pond water)

Pond or lake water known to support fungal growth is filtered through Whatman 541 filter paper. One part pond water is combined to two parts distilled water and autoclaved.

Haematoxylin and eosin staining

Before staining, sections must be completely de – waxed by placing in two changes of xylene each of 5-19 minutes duration. Sections are then transferred to water by first removing the xylene in absolute alcohol (74 O.P.) for 2-5 minutes and passing the sections through descending grades of alcohol e.g. 90%, 70%, 50% thus avoiding the possibilty of sections being removed from the slide by diffusion currents.

Procedure

- Take sections to tap water as described above, removing artefact pigments if necessary.
- Stain in haematoxylin for 5-20 minutes depending on which stain is used (Lendrum's iron or Mayers haematoxylin is recommended)
- Wash in running water for 2 minutes.
- Differentiate in 0.5% acid- alcohol for a few seconds. Check differentiation level by examination under the microscope.
- If the nuclei are sufficiently stained, "blue" in 2% potassium acetate (if Lendrum's haemotoxylin is used) for 5 minutes or alternatively in Scott's tap water substitute (see below) for 5 minutes.
- Wash sections in tap water.
- Stain in 1% alcoholic eosin for 3-5 minutes.
- Remove excess eosin by rinsing sections in absolutes alcohol (74 O.P.).
- Check counterstaining and if satisfactory clear in xylene.
- Mount in a synthetic resin medium.

Results:

      Nuclei: blue

      Cytoplasm, connective tissue, red blood cells and muscle: red/pink

Grocott's Modification of Gomori's Methamine Silver Method

(Gomori, 1946., Grocott, 1955)

Preparation:

Methenamine sliver nitrate

Methenamine silver nitrate stock solutions

5% Silver nitrate in distilled water	5ml.
3% methenamine in distilled water	100ml.

A white precipitate will form but this dissolves on shaking. Clear solutions will keep for some months in the refrigerator.

Methanamine silver nitrate working solution

5% Borax solution	2ml.
Distilled water	23ml.
Methenamine silver nitrate (stock solution)	25ml.

Technique

- Take sections to distilled water.
- Oxidize in 5% chronic acid (chromium trioxide) 1 hour.
- Wash in running tap water for a few seconds.
- Rinse briefly in 1% sodium bisulphate to remove residual chromic acid.
- Wash in tap water for 5 minutes.
- Wash with 3 or 4 changes of distilled water.
- Place in working methenamine silver nitrate solution in oven at 58C for 30-60 minutes. The section should be yellowish – brown (see Note below.)
- Rinse in 6 changes of distilled water.
- Tone in 0.1% gold chloride solution for 2-5 minutes.

- Rinse in distilled water.
- Place in 2% sodium thiosulphate (hypo) solution for 2-5 minutes to remove the unreduced silver.
- Wash thoroughly in water.
- Counterstain with light green solution (light green S.F. 0.2g., glacial acetic acid 0.2ml., distilled water 500ml.) for 20 seconds.
- Dehydrate, clear and mount in a synthetic resin medium.

Results:

> Fungi: sharply outlined in black
>
> Mucin: grey
>
> Background: pale green

Notes: After treatment with the silver solution, fungi should be dark brown in colour. It is advisable to check this with the microscope and a control section known to contain fungi should always be run at the same time.

Reticulin fibrils and threads of fibrin will be blackened by this method and must not be confused with fungi.

ANNEX 5

Table showing number of negative samples (recorded as 0), number dermally infected with *A.invadans,*(recorded as 1), and number positively identified as EUS (recorded as 2) within treatment groups.

Treatment	Number sampled	Number negative (as 0)	Number negative (as 1)	Number positive (as 2)	% positive
A invadans + Tapwater	33	11	7	14	42.4%
A invadans + Distilled water	38	15	4	19	50.0%
A invadans + pH 5 Distilled water	40	10	4	26	72.5%
A invadans + Tapwater + Scrape	8	2	1	5	62.5%
Tapwater	9	9	0	0	0
Distilled water	9	9	0	0	0
PH 5 Distilled water	7	3	0	4	57.1%

Annex 6

Results of Mycelium Minimum Inhibitory Concentration (MIC) test

Compound	Concentration (ppm)	Result 24Hrs	Result 48Hrs	Result 72Hrs	Result 96Hrs
D-limonene	25 ppm	+ ve	O	O	O
	250 ppm	+ ve	O	O	O
	2500 ppm	+ ve	O	O	O
	5000 ppm	+ ve	O	O	O
	10000 ppm	+ ve	O	O	O
D-limonene + E-Z-Mulse™	25 ppm	+ ve	O	O	O
	50 ppm	- ve	- ve	- ve	-ve
	100 ppm	- ve	- ve	- ve	-ve
	200 ppm	- ve	- ve	- ve	-ve
	250 ppm	- ve	- ve	- ve	-ve
E-Z-Mulse™	1ppm	- ve	+ ve	O	O
	10 ppm	+ ve	O	O	O
	20 ppm	+ ve	O	O	O
	25 ppm	-ve	-ve	-ve	+ve
	250 ppm	-ve	-ve	-ve	-ve
	2500 ppm	-ve	-ve	-ve	-ve
Commercial Neem Preparation	750 ppm	+ ve	O	O	O
	1500 ppm	- ve	- ve	+ ve	O
	3000 ppm	- ve	- ve	+ ve	O
	6000 ppm	- ve	- ve	+ ve	O
	7500 ppm	+ ve	O	O	O
	75000ppm	+ ve	O	O	O
Neem Seed Extract	750 ppm	+ ve	O	O	O
	7500 ppm	+ ve	O	O	O
	75000 ppm	+ ve	O	O	O
Neem Leaf Extract	750 ppm	+ ve	O	O	O
	7500 ppm	+ ve	O	O	O
	75000 ppm	+ ve	O	O	O
Malachite Green	1 ppm	- ve	- ve	+ ve	O

Annex 7

Results of zoospore production inhibition assay.

Compound	Concentration (ppm)	Result 24Hrs	Result 48Hrs
D-limonene	1 ppm	++	+++
	10 ppm	++	++
	100ppm	++	++
D-limonene + E-Z-Mulse™	1 ppm	++	++
	10 ppm	++	++
	100ppm	O	Few cysts
E-Z-Mulse™	1 ppm	++	++
	10 ppm	O	
	100ppm	O/	
Commercial Neem Extract	5 ppm	++	+++
	50 pp	O	O
	500 ppm	+	O
Neem Seed Extract	5 ppm	+++	+++
	50 pp	++	++
	500 ppm	++	++
Neem Leaf Extract	100 ppm	+++	++
	1000 ppm	+	++
	10000 ppm	+++	++